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Palindromic Adenovirus Type 5-Simian Virus 40 Hybrid

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A family of novel adenovirus type 5-simian virus 40 (Ad5-SV40) recombinants ($Ad5^{++}D1$) whose genomes consist of symmetrically inverted structures was isolated. Particles of $Ad5^{++}D1$ could contain one of several recombinant genomes that differed incrementally from one another by a full-length copy of linear SV40 DNA. The members of the $Ad5^{++}$ D1 family appeared to be in genetic equilibrium with one another. In all probability this equilibrium was maintained by homologous recombination, resulting in the loss or gain of one or two unit length copies of the SV40 genome. The genome of the most abundant recombinant form consisted of a giant inverted repeat which was some 35,000 nucleotide pairs in length. Beginning from one end, the recombinant genome consisted of 3,534 nucleotides derived from the left end of the adenovirus type 5 genome; these nucleotides were joined to 2.7 copies of SV40 DNA arranged as head-to-tail tandems. This entire structure was then repeated in the opposite orientation, thereby forming a large inverted repeat whose structure was Ad5-SV40-SV40-04VS-04VS-5dA. The population of hybrid genomes was stable and was maintained through serial rounds of infection.

Adenovirus-simian virus 40 (SV40) hybrids were isolated during the 1960s when human adenoviruses were grown in cultures of simian cells now known to have been contaminated with SV40. Human adenoviruses normally do not replicate in simian cells; however, for reasons that are not understood, the 80 amino acids comprising the C-terminal portion of the SV40 large T antigen carry a helper function that renders simian cells fully permissive to human adenoviruses (4). Although the genomes of these two viruses share no detectable homology, long-term cocultivation of SV40 and adenovirus allows the formation of recombinants whose genomes consist of SV40 sequences covalently attached to adenovirus DNA (1, 9).

Virtually all of our knowledge of the molecular biology of adenovirus-SV40 hybrids has come from analyses of the various components of a strain of virus called $Ad2^{++}$, which was derived in 1956 by passing adenovirus type 2 sequentially seven times in rhesus monkey kidney cells. As we now know, $Ad2^{++}$ contains wild-type adenovirus type 2, complete SV40 virions, and hybrids of many different sorts (17).

All adenovirus-SV40 hybrids studied so far have suffered a deletion of adenovirus sequences at the site of SV40 insertion. Depending on the length and genomic location of the deleted sequences, the hybrid may be nondefective and grow unassisted, or it may be defective and dependent on a helper adenovirus for growth. Those hybrids with the largest deletions of adenovirus often contain SV40 sequences arranged in the form of a tandem repetition (e.g., $Ad2^{++}$HEY and $Ad2^{++}$LEY) (10). Simian cells infected with such hybrids often yield infectious SV40, which originates by excision from the hybrid genome. The mechanism by which excision occurs is unknown, but the simplest hypothesis is that the free SV40 genomes arise by intramolecular, homologous recombination between elements of the SV40 tandem array (10). However, the existence of the tandem array also allows a different form of recombination to occur, namely, unequal cross-over between hybrid genomes. Such recombinational events yield two new hybrids, which have one less and one more SV40 genome than the parent molecules. Thus, the hybrids with tandem arrays of SV40 DNA exist as populations whose components are in genetic equilibrium with one another (18).

In different hybrids, the SV40 DNA can be carried in the adenovirus genome in either of the two possible orientations and may be inserted in either the early or late adenoviral genes. A comparison of the structures of different natural hybrid DNAs by restriction endonuclease digestion or heteroduplex analysis has shown that the junctions between SV40 and adenovirus DNA can occur in many different locations in both viral genomes.

In this paper we describe the isolation of a novel adenovirus type 5-SV40 (Ad5-SV40) hy-

brid ($Ad5^{++}D1$), which is different from any adenovirus-SV40 hybrid so far described but is similar in structure to a host-substituted adenovirus type 12 genome (3). The genome of $Ad5^{++}D1$ consists of a symmetric inverted repeat which is some 35,000 base pairs in length.

MATERIALS AND METHODS

Cells. CV-1 cells, a line of African green monkey kidney cells, and HeLa cells, a line of human carcinoma cells, were cultured in plastic dishes in the Dulbecco modification of Eagle medium as described previously (7).

Viruses. A population of Ad5-SV40 hybrid viruses (seventh passage of adenovirus type 5 in rhesus monkey kidney cells) was a gift from W. P. Rowe. The adenovirus type 5 used as wild type in these experiments was isolated from this stock as described below. Plaque titrations of adenovirus type 5 were performed as described by Williams (20) on monolayers of HeLa cells. Plaque assays of Ad5-SV40 hybrid virus were performed on CV-1 monolayers. Plaques generally developed on both cell types after incubation for 5 to 7 days at 37°C.

Large quantities of the defective Ad5-SV40 hybrids were prepared by infection of CV-1 cells in plastic dishes (30 to 40 by 90 mm; Falcon Plastics). After 54 h at 37°C, the cells were washed with ice-cold phosphate-buffered saline and concentrated by centrifugation. Virus particles were isolated as described by Lonberg-Holm and Philipson (11) and then purified by CsCl buoyant density centrifugation.

Isolation of viral DNA. Intracellular DNAs of adenovirus type 5 or $Ad5^{++}D1$ were extracted from infected CV-1 cells 30 h postinfection by a modified Hirt procedure (8). Cells were incubated at 37°C for 2 h with lysis buffer (0.6% sodium dodecyl sulfate, 10 mM EDTA) containing 1 mg of pronase per ml. NaCl was added to final concentration of 1 M, and after 4 h or more on ice, viral DNA was separated from cellular DNA by centrifugation at 20,000 rpm for 30 min at 4°C.

The DNAs of adenovirus type 5 and $Ad5^{++}D1$ were isolated from purified virus particles as described by Pettersson and Sambrook (14). SV40 DNA was isolated by the Hirt procedure (8), and component I DNA was purified by ethidium bromide-CsCl gradient centrifugation.

Snapback of viral DNA. DNA was denatured in 80% formamide–3× TE (30 mM Tris, 3 mM EDTA) at 68°C for 5 min (10 μg in 0.35 ml). The solution was then adjusted to 50% formamide–3× TE and was placed on ice for 30 min to allow snapback of the DNA to occur.

S1 nuclease digestion. Viral DNA treated as described above was digested for 30 min at 37°C with 30 U of S1 nuclease in 0.5 ml of solution containing 0.25 M NaCl–0.03 M sodium acetate (pH 4.6)–0.001 M $ZnSO_4$.

Restriction enzyme analysis. DNAs were digested with restriction endonucleases *Bam*HI, *Hae*II, *Tac*I, *Eco*RI, *Bgl*I, *Taq*I, *Hpa*II, *Sma*I, *Xba*I, *Hin*dIII, *Sst*I, *Kpn*I, and *Bgl*II by using previously published protocols (New England Biolabs or Bethesda Research Laboratories). The products of digestions were subjected to electrophoresis on horizontal 0.7 or 1.0% agarose gels and transferred to nitrocellulose filters by the method of Southern (15). Filters were hybridized to ^{32}P-labeled DNA probes prepared by nick translation with four α-^{32}P-labeled deoxynucleoside triphosphates as described previously (12) at 68°C for 6 to 24 h. The filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) containing 1% sodium dodecyl sulfate at 68°C for 1 to 2 h, and autoradiographs were then made.

Preparation of nitrocellulose filters for rehybridization. ^{32}P-labeled DNA probes were removed from filters which were to be rehybridized by treatment with a solution containing 50% formamide, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), 3× SSC, and 100 μg of salmon sperm DNA per ml at 68°C for 1 h. The nitrocellulose filters were then washed with 2× SSC and hybridized to the appropriate ^{32}P-labeled DNA probe.

RESULTS

Isolation of $Ad5^{++}D1$. The scheme used to isolate $Ad5^{++}D1$ is shown in Fig. 1. Preparation 30498 was a virus stock obtained by W. P. Rowe and associates in 1956 by passing adenovirus type 5 sequentially seven times in primary cultures of rhesus monkey kidney cells. When we received the preparation in 1978, its titer was very low on both simian (CV-1) and human (HeLa) cells. In fact, the only virus that could be detected easily was SV40. Therefore, the original stock was amplified by two sequential passages in HeLa cells, a regimen that was expected to increase the titers of the parental adenovirus type 5 and any nondefective hybrid viruses and to select against SV40 and defective hybrid viruses. This stock contained adenovirus type 5 without nondefective hybrids, as shown by the appearance of plaques on HeLa cells but not on CV-1 cells. The virus stock derived in this way was then used as a helper for the growth of preparation 30498 in CV-1 cells. The resulting stock was assayed by plaque formation on monolayers of CV-1 cells in the presence and absence of SV40 antiserum and also on HeLa cells. Plaques with typical adenovirus morphology appeared after 5 days and were counted after 8 days of incubation. Plaques with typical SV40 morphology appeared and were counted 3 days later. Most of the stock consisted of adenovirus type 5, and most of the virus capable of forming plaques on monolayers of CV-1 cells was SV40. However, 1% of the plaques arising after 5 days of incubation on CV-1 cells were adenovirus-like in their morphology and were not inhibited by SV40 antiserum. Individual plaques were picked and used to prepare virus stocks. Cultures of CV-1 cells were infected with these viruses, and after incubation for 35 h viral DNA was extracted by the method of Hirt (8). Viral DNAs were analyzed by restriction enzyme digestion, gel electrophoresis, and Southern hybridization. Six stocks were examined, and these six stocks yielded identical results. An analysis of two of these stocks is shown in Fig. 2. There were at

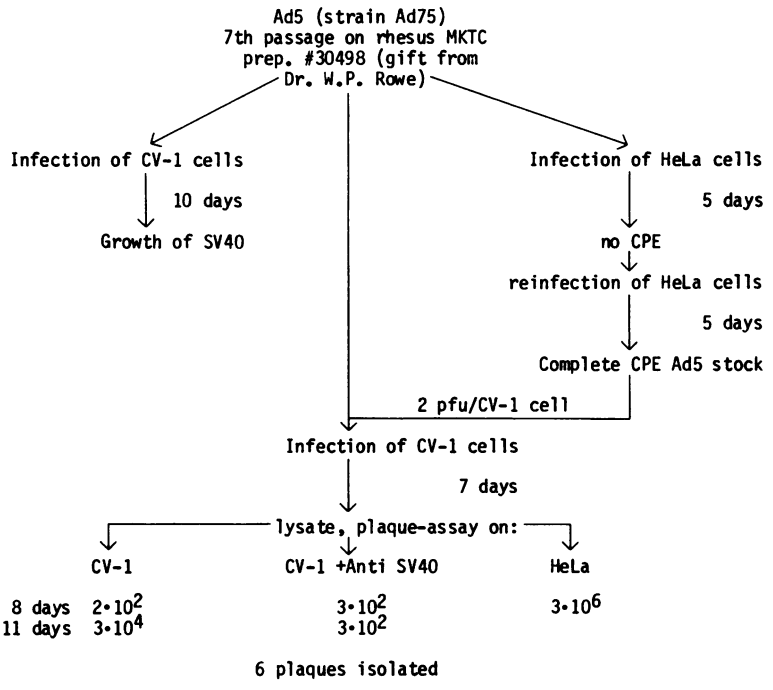

FIG. 1. Scheme used to isolate Ad5^{++}D1. To increase the titer of the wild-type adenovirus type 5, the original virus stock was passed twice on HeLa cells and then used as helper with the original stock to infect CV-1 cells. The lysate from this infection was assayed for plaque formation on CV-1 and HeLa cells and on CV-1 cells with SV40 antiserum. Plaques exhibiting adenovirus morphology appeared 5 days after infection and were counted after 8 days. SV40 plaques appeared and were counted 3 days later. Six plaques that appeared on CV-1 cells in the presence of SV40 antiserum were isolated and analyzed. Ad5, Adenovirus type 5; CPE, cytopathic effects; MKTC, monkey kidney tissue culture.

least five bands present in the undigested hybrid DNA, all of which contained SV40 sequences, as shown by hybridization to a radioactive SV40 DNA probe. Digestion with restriction endonuclease *Bam*HI or *Hae*II yielded bands in tracks containing hybrid DNAs that were not detectable in tracks containing adenovirus type 5 DNA. These bands hybridized specifically to a radioactive SV40 DNA probe. Figure 3 shows DNA isolated from hybrid virions analyzed in a similar manner. Digestion with endonuclease *Tac*I, *Eco*RI, or *Bgl*I yielded bands which were not present in the adenovirus type 5 DNA tracks and which hybridized to SV40 DNA. In the case of the *Eco*RI and *Bgl*I digests (both cut SV40 DNA once), one of the novel bands comigrated with unit length, linear SV40 DNA. When the hybrid DNA was digested with *Tac*I, an enzyme that cleaves adenovirus type 5 DNA more than 50 times but does not cut the SV40 genome, a series of bands was produced, the largest of which was equivalent in length to about 80% of adenovirus type 5 DNA. The other bands migrated through the gel as a ladder whose members differed in length by roughly integral multiples of one linear SV40 genome. These data are reminiscent of those obtained with Ad2^{++}HEY (10), and they suggest that the Ad5^{++}D1 hybrid population is heterogeneous and consists of viruses whose genomes contain tandem insertions of SV40 and differ from each other by one unit length of SV40 DNA.

The genetic stability of the Ad5^{++}D1 population was determined by further subcloning of one of the original six plaque-purified virus stocks. All derivatives of this stock that were examined were found to contain populations of DNA identical to the populations of the parental isolates. Along with the observation that no alteration in genome structure was detected during serial passage, this result suggests that Ad5^{++}D1, like Ad2^{++}HEY and Ad2^{++}LEY (10, 18), consists of a population of hybrids in genetic equilibrium with one another.

SV40 sequences in the AD5^{++}D1 genome. To facilitate mapping of the SV40 sequences, Ad5^{++}D1 DNA was first digested with *Tac*I, an enzyme that cleaves adenovirus type 5 DNA more than 50 times but does not have a recognition site within the SV40 genome. The products of this initial digestion were then cleaved with a variety of endonucleases that are known to cut

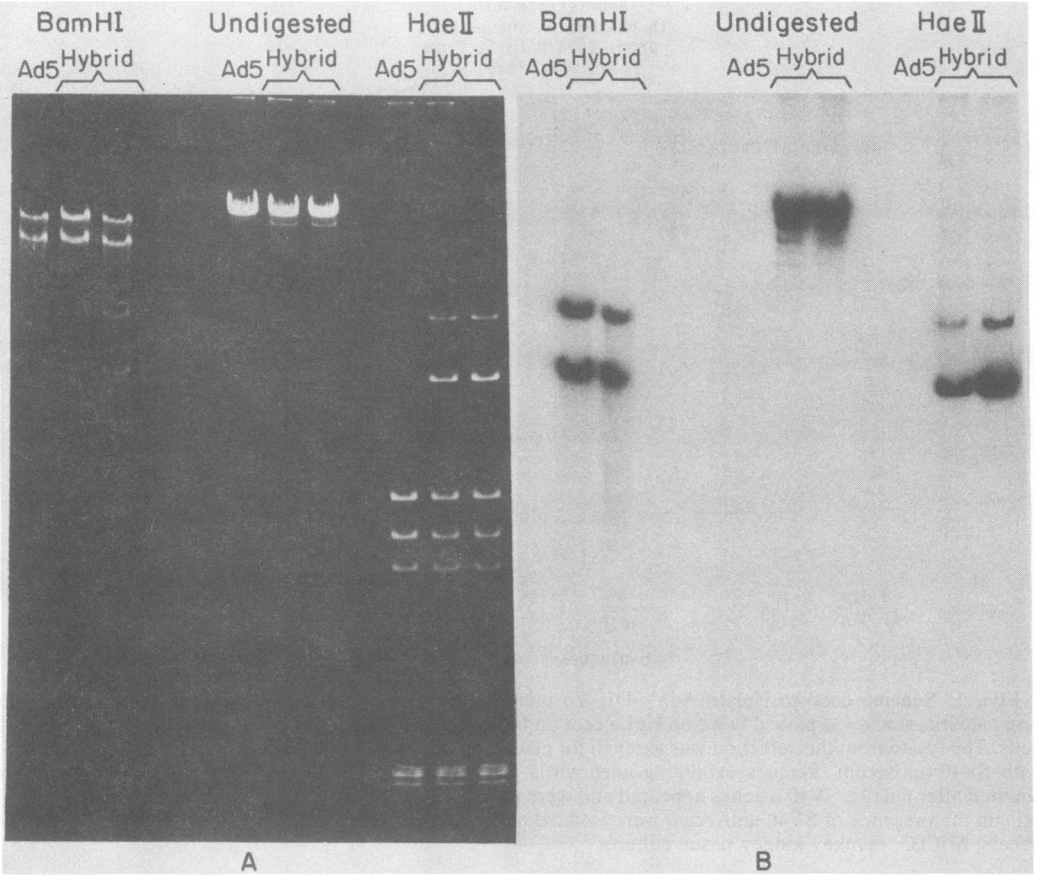

FIG. 2. Intracellular viral DNA was isolated from cells infected with Ad5^{++}D1 by the Hirt method (8), digested with *Bam*HI or *Hae*II, separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled SV40 DNA. (A) Ethidium bromide-stained gel. (B) Pattern of hybridization to SV40 DNA. Ad5, Adenovirus type 5 DNA; hybrid, two separate plaque isolates of Ad5^{++}D1 DNA.

SV40 DNA once (Fig. 4). Each enzyme yielded three types of fragments that hybridized to ^{32}P-labeled SV40 DNA. (i) One type was a fragment that hybridized only to SV40 DNA and migrated through gels at a rate indistinguishable from the rate of migration of unit length linear SV40 DNA. This result indicated that at least some of the SV40 genomes in the hybrid DNA were arranged as head-to-tail tandems. (ii) A second type was small fragments that hybridized to SV40 DNAs. Presumably these fragments represented the junctions between the viral DNAs. However, it was not possible to determine whether these fragments hybridized to adenovirus DNA because of the background of fragments produced by *Tac*I digestion of the adenovirus type 5 helper DNA. (iii) The third type was a fragment whose size varied according to the restriction enzyme used and which hybridized only to SV40 DNA. These fragments formed a series whose molecular weights increased as a function of twice the distance between the known site of cleavage and position 0.09 on the SV40 genome. This result suggested that each member of this series of fragments consisted exclusively of SV40 DNA sequences arranged in the form of an inverted repeat. The axis of symmetry within each of these fragments mapped at position 0.09 on the SV40 genome. These observations led to the map of the SV40 insert shown in Fig. 4. Note that digestion of the hybrid genome with *Bam*HI (Fig. 4) yielded a fragment of about 600 base pairs that was not detected in the experiment described above. The existence of this fragment was verified by using a different experimental approach. Ad5^{++}D1 DNA was digested with *Bam*HI, fragments were labeled with polynucleotide kinase and [γ-^{32}P]ATP and separated on an agarose gel, and bands were visualized by autoradiography (data not shown).

Adenovirus type 5 DNA sequences in the

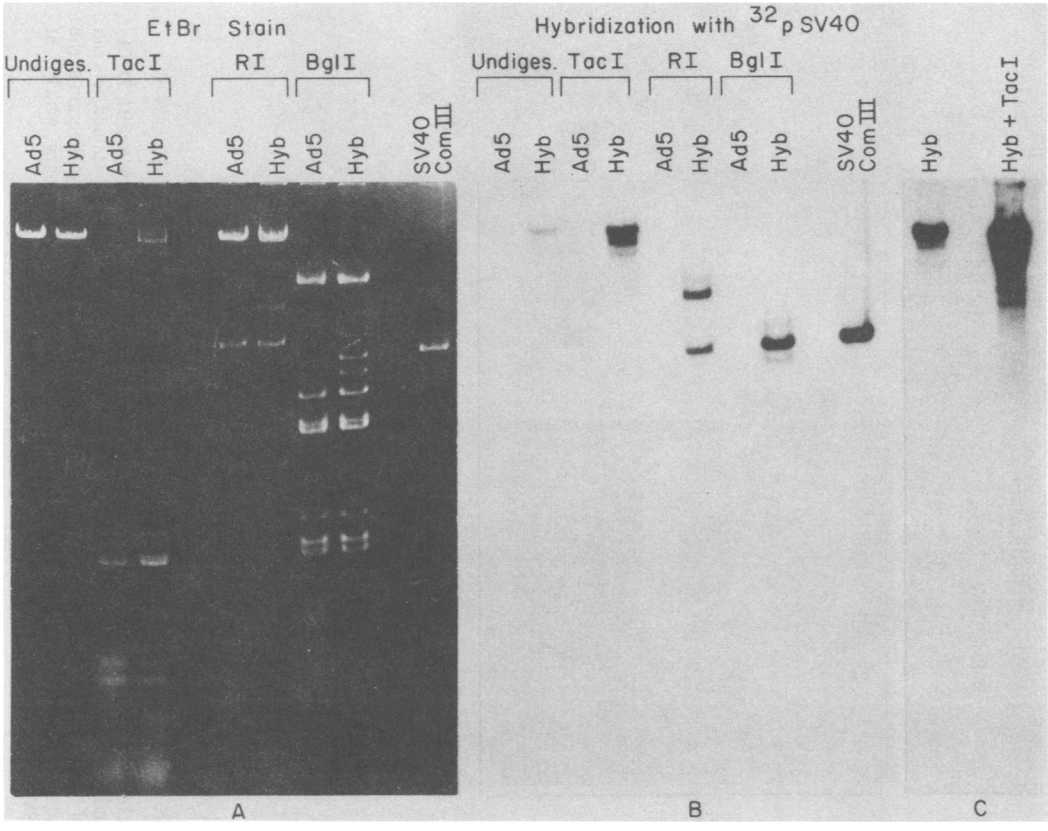

FIG. 3. Ad5^{++}D1 virions were purified by centrifugation to equilibrium in CsCl gradients. DNA was extracted from the virions with 0.5% sodium dodecyl sulfate and 1 mg of pronase per ml, digested with endonucleases *Tac*I, *Eco*RI (RI), and *Bgl*I, separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled SV40 DNA. (A) Ethidium bromide (EtBr)-stained gel. (B) Corresponding hybridization pattern. (C) Longer exposure of the undigested (Undiges.) and *Tac*I-digested DNA tracks of (B), showing the various forms of the hybrid virus (Hyb). Ad5, Adenovirus type; SV40 ComIII, SV40 component III DNA.

Ad5^{++}D1 genome. To identify the adenoviral sequences within the hybrid virus genome, Ad5^{++}D1 DNA was cleaved with restriction endonucleases, and the resulting fragments were separated by gel electrophoresis, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled DNA probes. Some filters were hybridized sequentially to different probes as described above.

Cleavage of the recombinant genomes with *Eco*RI and *Bam*HI yielded three and two bands, respectively, which were not present in digested adenovirus type 5 DNA. In each case, only one of the fragments hybridized both to labeled SV40 DNA and to adenovirus DNA (Fig. 5); therefore, this fragment must have contained a junction between the two viral DNAs. The *Bam*HI fragment of the Ad5^{++}D1 genome containing the junction was purified by gel electrophoresis, nick translated by using ^{32}P-labeled deoxynucleoside triphosphates, and hybridized to a nitrocellulose filter containing fragments of adenovirus type 5 DNA generated by digestion with *Sma*I, *Hin*dIII, or *Xba*I (Fig. 6). We observed hybridization to all fragments that mapped on the adenovirus type 5 genome to the left of map position 0.11; no hybridization was detected to fragments derived from the remainder of the genome. These data suggest that Ad5^{++}D1 (i) contains only one junction between adenovirus sequences and SV40 sequences and (ii) contains adenovirus sequences that are derived exclusively from the left end of the adenovirus type 5 genome. These sequences must extend through position 0.079 (the junction of *Hin*dIII fragments G and E) but can go no further than position 0.11 (the junction of *Sma*I fragments F and N). Therefore, the junction fragment between adenovirus type 5 and SV40 DNAs contains between 2,765 and 3,885 nucleotides of adenovirus type 5 sequence.

To verify the symmetric structure of the hy-

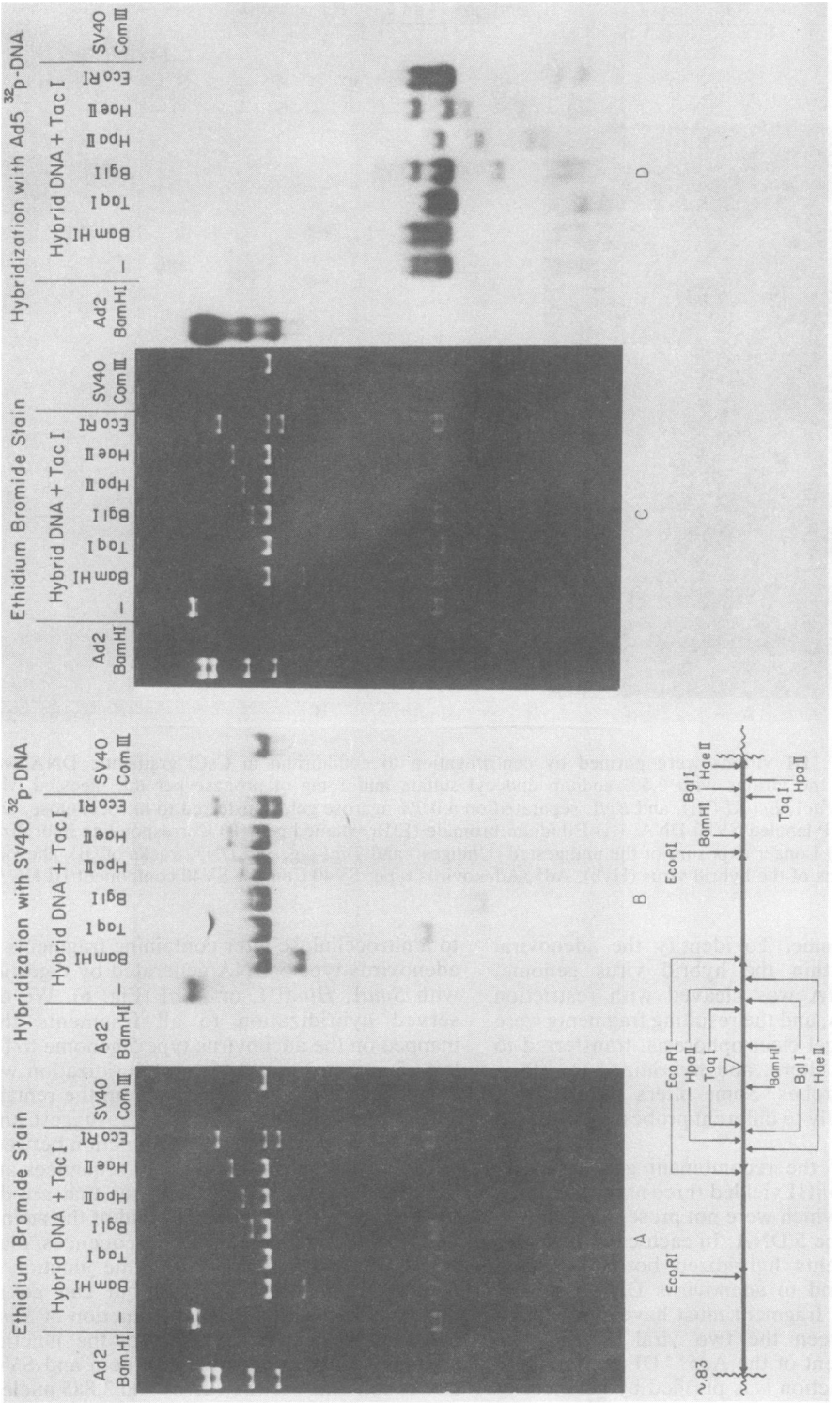

FIG. 4. DNA purified from virions was digested first with endonuclease *Tac*I and subsequently with either *Bam*HI, *Taq*I, *Bgl*I, *Hpa*II, *Hae*II, or *Eco*RI. DNAs were separated on 0.7% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled DNA probes. (A and C) Identical ethidium bromide-stained gels run in parallel. (B) Pattern of hybridization to ^{32}P-labeled SV40 DNA. (D) Pattern of hybridization to ^{32}P-labeled adenovirus type 5 DNA. The schematic diagram at the bottom shows the cleavage sites of the various enzymes utilized and the fragments that could result. The open triangle marks the center of symmetry, which is at about 0.09 map unit on the genome. Ad2, Adenovirus type 2; SV40 ComIII, SV40 component III DNA; Ad5, adenovirus type 5.

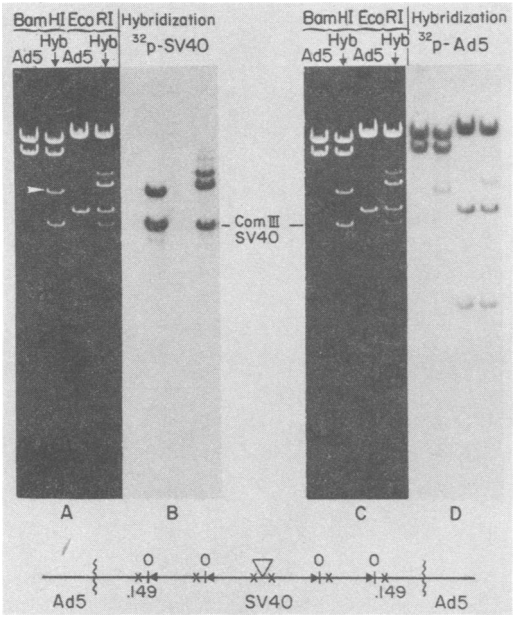

FIG. 5. Ad5^{++}D1 and adenovirus type 5 (Ad5) DNAs were digested with endonucleases *Bam*HI and *Eco*RI, and the fragments were separated on 0.7% agarose gels and transferred to nitrocellulose. One filter was hybridized to ^{32}P-labeled SV40 DNA, and the other was hybridized to ^{32}P-labeled adenovirus type 5 DNA. (A and C) Ethidium bromide-stained gels that were run in parallel. (B and D) Patterns of hybridization to ^{32}P-labeled SV40 DNA and ^{32}P-labeled adenovirus type 5 DNA, respectively. The diagram at the bottom indicates the structure of the hybrid virus (Hyb), showing the sites of cleavage for *Eco*RI (O) and *Bam*HI (×). (A) The white arrowhead indicates the band in the *Bam*HI digest of hybrid DNA that hybridized to both probes. The *Eco*RI fragment that hybridized to both probes was slightly larger than this *Bam*HI fragment. ComIII SV40, SV40 component III DNA.

brid genome, Ad5^{++}D1 DNA was denatured with formamide and allowed to renature into snapback structures as described above. Samples of DNA were then treated with S1 nuclease alone, endonuclease *Tac*I alone, or S1 nuclease followed by *Tac*I. The resulting fragments of DNA were separated by gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled SV40 DNA. After autoradiographs were made (Fig. 7), the SV40 probe was removed from the nitrocellulose with formamide, and the DNA on the filter was then hybridized to ^{32}P-labeled adenovirus DNA. The data described below relate to the most prominent bands in Fig. 7. The minor bands all behaved as predicted from the structure of Ad5^{++}D1. Native Ad5^{++}D1 DNA had a size identical to that of wild-type adenovirus type 5 DNA. Denatured and renatured DNA (snapback DNA) was approximately one-half as large as wild-type adenovirus type 5 DNA, a result which is consistent with the idea that the hybrid genome is an inverted repeat. Treatment of snapback DNA with S1 nuclease did not detectably alter the size of the molecule, verifying that the majority of this DNA must consist of double-stranded sequences. From the data shown in Fig. 5 and 6, we predicted that treatment of native Ad5^{++}D1 DNA with *Tac*I would reduce its size by approximately 20%. Under the conditions used, this difference was not resolvable. We also predicted that treatment of snapback DNA with *Tac*I would reduce its size by 10% (from 50 to 40% of adenovirus type 5 DNA). This shift is clearly shown in Fig. 7. These data indicate that the SV40 DNA sequences are located at the center of the Ad5^{++}D1 genome, and they provide further evidence that the genome of the hybrid virus is an inverted repeat, with adenovirus DNA sequences flanking multiple copies of SV40 sequences, as shown in Fig. 5.

This structure was confirmed by the data in Fig. 8. Figure 8A shows the pattern of hybridization of ^{32}P-labeled adenovirus type 5 DNA to a Southern transfer of Ad5^{++}D1 DNA which had been denatured, allowed to snapback, treated with S1 nuclease, and then digested with *Sst*I or *Kpn*I. Also shown are the results of digestions of adenovirus type 5 DNA with the same restriction enzymes. Figures 8B and C show the same nitrocellulose filter hybridized to ^{32}P-labeled adenovirus type 5 DNA or a purified fragment of DNA derived from the left end of the adenovirus type 5 genome (0 to 0.094 map unit). The junction fragment in each digest that hybridized to both adenovirus DNA and SV40 DNA is indicated (Fig. 8A through C, asterisks). The fragment that contained the center of symmetry of Ad5^{++}D1 and hybridized to SV40 DNA is also indicated (Fig. 8A, ×). In addition, we detected a fragment in the *Kpn*I digest corresponding to unit length linear SV40 DNA (not marked). Both adenovirus DNA probes hybridized only to fragments from the left end of the adenovirus genome (Fig. 8B and C), and these fragments (fragments H and A) are indicated in Fig. 8A through C and also in the schematic representation of the restriction map of the leftmost 23.5% of the adenovirus type 5 and Ad5^{++}D1 genomes shown at the bottom of Fig. 8. These data are consistent only with the structure shown in Fig. 5.

Sequence of the junction between adenovirus type 5 and SV40 DNAs. A *Bgl*II-*Bam*HI fragment of the genome of the hybrid virus containing the junction was cloned into the *Bam*HI site of pBR322. Closed circular plasmid DNA was isolated, digested with endonuclease *Hin*dIII, and

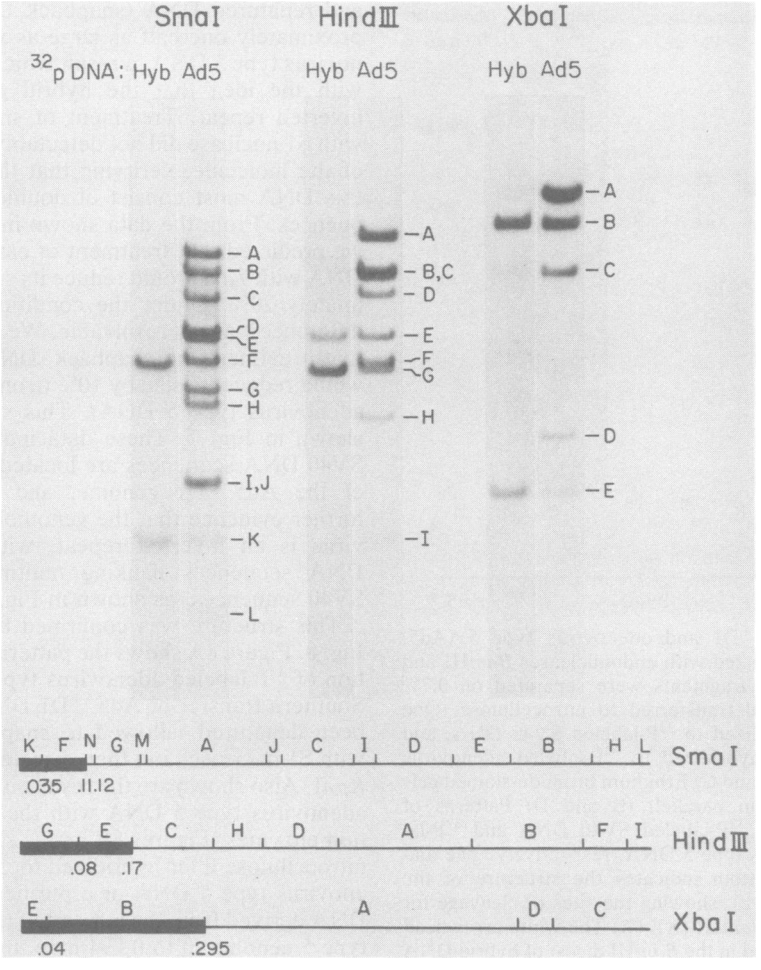

FIG. 6. Adenovirus type 5 (Ad5) DNA was digested with endonucleases *Sma*I, *Hin*dIII, and *Xba*I, separated on a 1% agarose gel, and transferred to nitrocellulose. The *Bam*HI fragment of Ad5^{++}D1 containing the junction of the viral DNAs (see Fig. 5) was purified by gel electrophoresis, nick translated with ^{32}P-labeled deoxynucleoside triphosphates, and used to probe the nitrocellulose filters. Restriction maps of the adenovirus type 5 genome for each enzyme used are illustrated at the bottom. Each line represents the adenovirus type 5 genome, and the solid boxes correspond to the portions that hybridized to the ^{32}P-labeled junction fragment probe. Hyb, Hybrid virus.

end-labeled with [α-^{32}P]dATP by using the Klenow fragment of *Escherichia coli* DNA polymerase I. The labeled DNA was then digested with endonuclease *Eco*RI, and the resulting fragments were separated by gel electrophoresis. The asymmetrically labeled fragment containing the junction was then isolated and sequenced by the method of Maxam and Gilbert (13) (Fig. 9). The adenovirus type 5 sequences present in Ad5^{++}D1 ended at nucleotide 3,534. SV40 sequences began at nucleotide 1,102 and extended counterclockwise around the genome to about map unit 0.09. Between the adenovirus type 5 and SV40 sequences the dinucleotide AA was found (Fig. 10). We cannot say which viral genome donated this dinucleotide to the hybrid since both contained this sequence at this position.

DISCUSSION

We have described the arrangement of the genome of a newly isolated adenovirus type 5-SV40 hybrid. This genome is a symmetric structure in which sequences derived from the left terminus of adenovirus type 5 DNA flank 5.5 copies of the SV40 genome as shown in Fig. 11. These SV40 DNA sequences are arranged in the form of two head-to-tail tandems, approximately 2.7 genomes in length, which point from different directions toward an axis of symmetry at the

center of the genome. Therefore, the entire DNA molecule is arranged in the form of a giant inverted repeat which is some 35,000 nucleotides in length. Deuring et al. (3) have described a similar structure that consists of repetitive sequences of human DNA flanked by some 700 to 1,100 nucleotides derived from the left end of the adenovirus type 12 genome. By contrast, Ad5^{++}D1 contains 3,534 base pairs of adenovirus type 5 DNA at each end of its genome. This region of the adenovirus type 5 chromosome contains the sequences necessary for transformation of cells (early region 1a and part of early region 1b), an origin of DNA replication (21), and packaging signals (5, 6). Early region 1a is also an important control region and specifies products that are necessary for the expression of the remainder of the viral genome. It is conceivable that by carrying two copies of this region of the adenovirus type 5 genome Ad5^{++}D1 gained some selective advantage over other Ad5-SV40 hybrids that might have been present in the original monkey-adapted population, either at the level of DNA replication, in control of expression of the genome, or in packaging of the viral genome into particles.

Throughout our experiments, DNA was prepared and examined on many separate occasions. Each preparation yielded the same pattern of bands, indicating that the Ad5^{++}D1 population is stable with respect to deletions and rearrangements of the genome. When intracellular DNA prepared by the Hirt method was compared with DNA prepared from virions, we

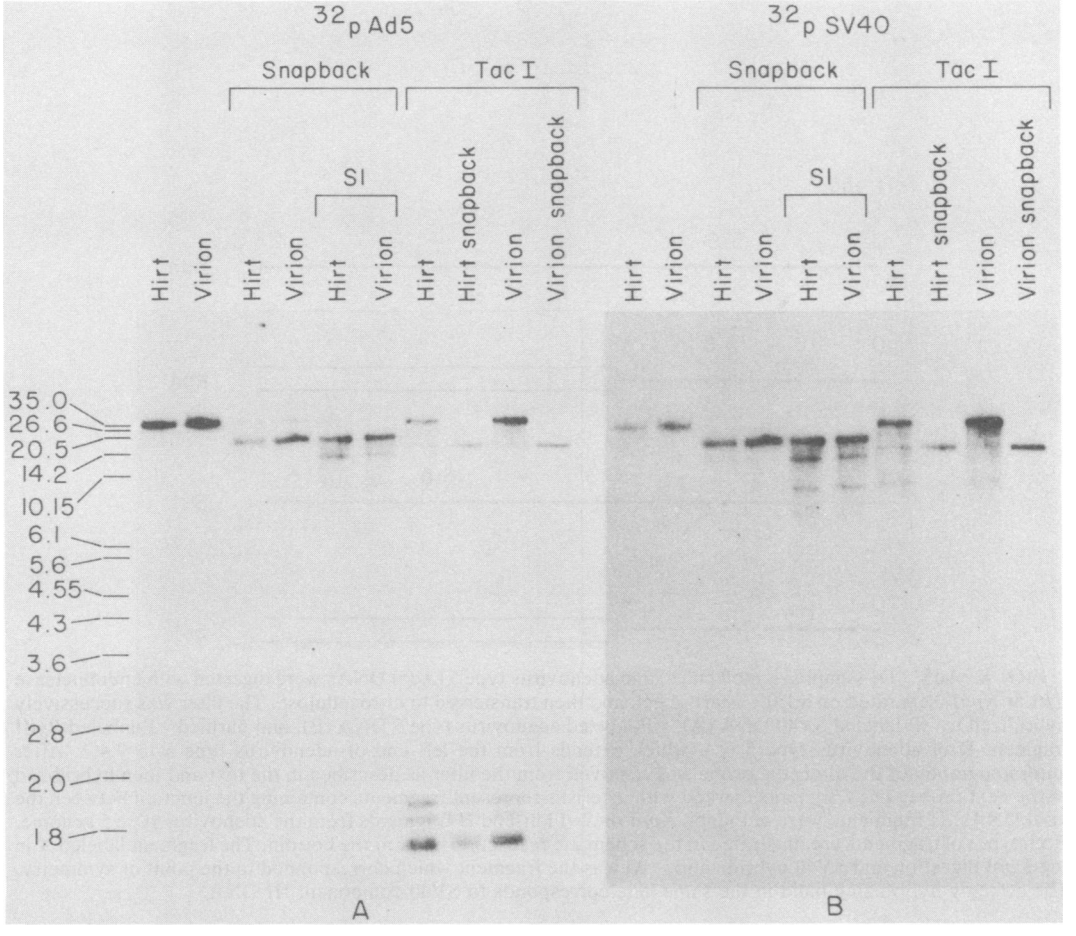

FIG. 7. Viral DNAs purified from virions or by Hirt extraction were left untreated, formamide denatured (10 μg in 0.35 ml), and allowed to snapback; then they were treated with S1 nuclease alone, *Tac*I alone, or *Tac*I after treatment with S1 nuclease. The resulting DNAs were separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled adenovirus DNA probe (A). The probe was then denatured from the DNA on the nitrocellulose filter as described in the text and hybridized to a ^{32}P-labeled SV40 DNA probe (B). Ad5, Adenovirus type 5.

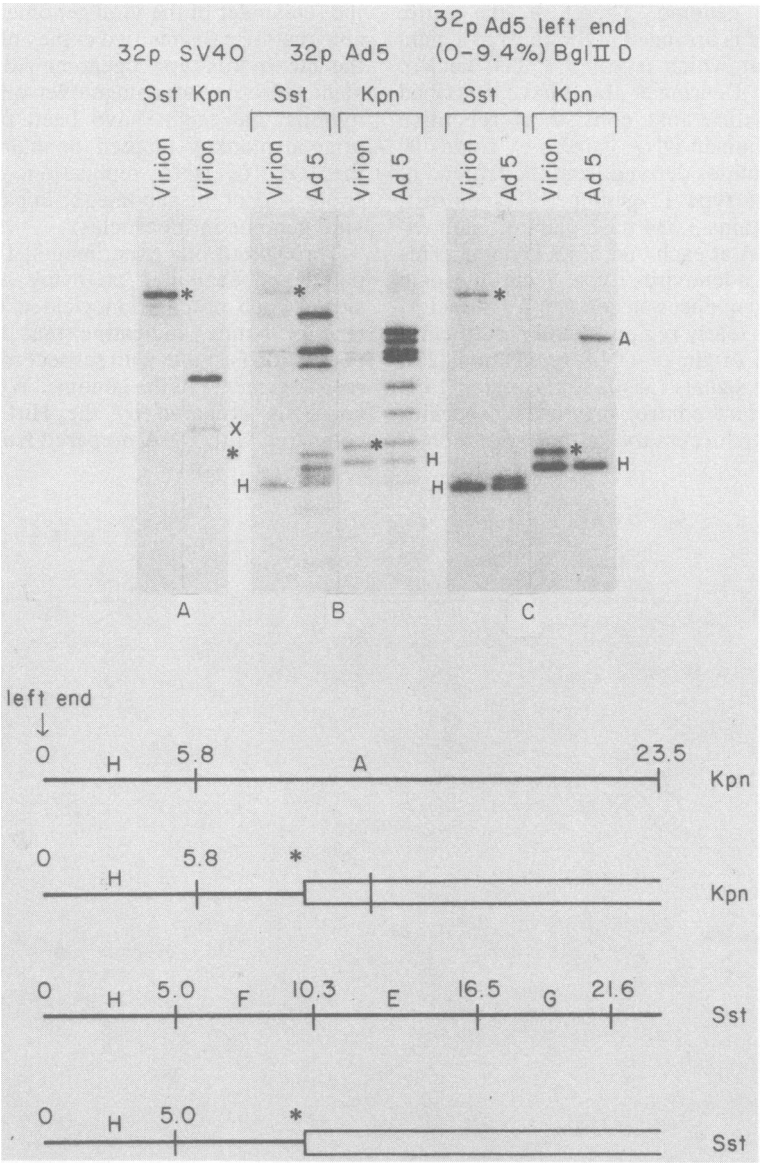

FIG. 8. Ad5^{++}D1 (snapback molecules) and adenovirus type 5 (Ad5) DNAs were digested with endonuclease *Sst*I or *Kpn*I, separated on a 1.0% agarose gel, and then transferred to nitrocellulose. The filter was successively hybridized to ^{32}P-labeled SV40 DNA (A), ^{32}P-labeled adenovirus type 5 DNA (B), and purified ^{32}P-labeled *Bgl*II fragment D of adenovirus type 5 (C), which extends from the left end of adenovirus type 5 to 9.4%. After autoradiography of the filter, the probe was removed from the filter as described in the text and then hybridized to the next probe. The fragments marked with asterisks represent fragments containing the junction between the viral DNAs. H fragments were authentic *Kpn*I or *Sst*I left-end H fragments from the adenovirus type 5 genome. Both types of fragments are illustrated in the schematic restriction maps at the bottom. The fragment labeled X in the *Kpn*I digestion and SV40 hybridization (A) was the fragment which corresponded to the point of symmetry. The strongly hybridizing band in the same lane corresponds to SV40 component III DNA.

found slight differences in the proportions with which the various members of the Ad5^{++}D1 family were represented. DNA prepared from virions showed a predominance of the species that had 5.5 copies of SV40 DNA in the insertion. This was also the most abundant species in intracellular DNA, although the variance in abundance was much less dramatic. Therefore, it seems that the longer form of the hybrid genome is preferentially packaged into virions.

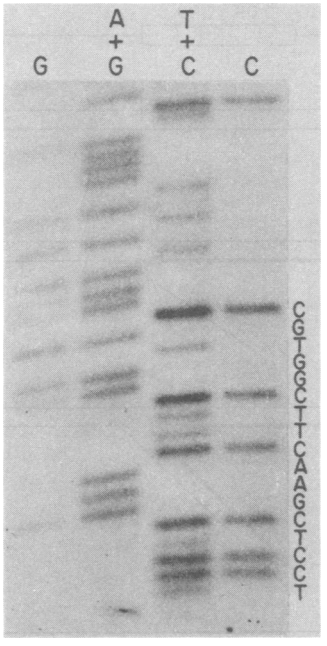

FIG. 9. Sequence of the junction fragment of Ad5^{++}D1. pAdSVJ-6 DNA was digested with *Hin*dIII and end-labeled by using [α-^{32}P]dATP and the Klenow fragment of *E. coli* DNA polymerase I. Labeled DNA was digested with *Eco*RI and separated on a 1.2% agarose gel (low melting temperature), and the largest fragment was isolated. DNA was sequenced as described by Maxam and Gilbert (13).

When we looked further at the forms of hybrid DNA present in virions, we found that the hybrid DNAs containing an odd number of SV40 genomes (i.e., 5.5, 3.5, or 1.5 copies) were represented more frequently than the hybrid DNAs that contained an even number of SV40 genomes (4.5 or 2.5 copies). This result suggests that there is some preference in packaging for hybrid DNA which is of a certain size or is entirely symmetric in structure. At present we do not understand the factors that are involved in this phenomenon.

The adenovirus type 5 and SV40 DNA sequences around the junction sites in the hybrid genome were examined for any sequence homology which might exist. Five of six nucleotides at the junction were identical in the two viral genomes (Fig. 10). Although this amount of homology is small, its proximity to the junction is striking. A similar degree of homology has been observed between SV40 DNA and an unoccupied site of integration in the rat genome (16). Therefore, it is possible that such small regions of homology are sufficient to bring DNA molecules in mammalian cells infected with SV40 into a position suitable for recombination. However, no homologies have been found at the junctions of at least five other adenovirus-SV40 hybrids that have been examined. Furthermore, in all cases analyzed (Ad2^{+}ND1dp1,2,3 [J. Sambrook, unpublished data]; Ad2^{++}HEY, Ad2^{++}LEY, and Ad2^{+}D1 [19, 22; L. Ling, M. Manos, and Y. Gluzman, submitted for publication]), the nucleotide sequences of SV40 DNA on one side of the junction have no homology with the adenovirus sequences on the other. All of these data indicate that adenovirus-SV40 hybrids were generated by illegitimate recombinational events between the two viral DNAs much like those which seem to occur during integration of SV40 or adenovirus DNA into the genomes of cells (17).

The symmetric structure of the Ad5^{++}D1 genome is so unusual and complex that we imagine that it was not the initial product of recombination between adenovirus type 5 DNA and SV40 DNA, but rather was generated from a progenitor adenovirus-SV40 hybrid genome. The parental molecule would necessarily have contained the junction between nucleotide 3,534 of adenovirus type 5 DNA and nucleotide 1,102 of SV40 DNA. SV40 sequences would have extended through 0.09 map unit into an intact, unit length, linear copy of the SV40 genome. The proposed parental molecule might then have returned into the adenovirus sequences, or it may have contained still another copy of linear SV40 DNA. The proposed model by which the Ad5^{++}D1 genome might have been generated is

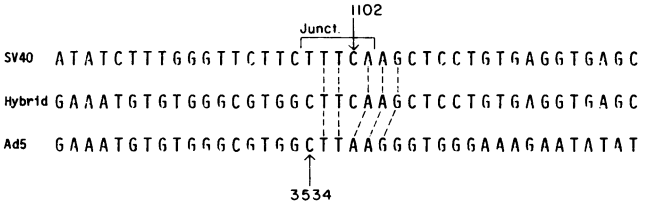

FIG. 10. Very limited sequence homology was observed at the junction of the viral DNAs, as indicated by the dashed lines. The adenovirus type 5 (Ad5) sequences end at position 3,534, and SV40 begins at nucleotide 1,102. It is impossible to say which viral genome contributed the dinucleotide TT at the center of the junction since this structure is present in both viral genomes.

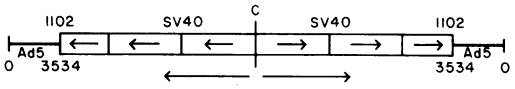

FIG. 11. Structure of the recombinant genome of Ad5^{++}D1. The genome is comprised of 3,534 nucleotides from the left end of the adenovirus type 5 (Ad5) genome covalently joined to SV40 DNA at nucleotide 1,102. There are approximately 2.7 copies of SV40 DNA, which are then inverted, and the entire structure is repeated. The adenovirus sequences are indicated by the lines, and the SV40 sequences are indicated by the boxes. The center of symmetry (C) is indicated and resides at about 0.09 map unit on the SV40 genome. The SV system (17) of numbering for SV40 is used throughout.

shown in Fig. 12. The parental adenovirus-SV40 hybrid molecule would initiate replication from the origin at the conventional left end of the adenovirus genome (Fig. 12C). Replication would continue through the adenoviral sequences and into the SV40 sequences. At some point while the growing replication fork was still within the SV40 DNA, we propose that the polymerase switched strands and began to replicate the complementary strand of DNA, as indicated in Fig. 12D. A second round of replication could then begin (Fig. 12E), resulting in the formation of (i) a newly replicated parental molecule (Fig. 12F) and (ii) the symmetric product of the template switching event, which would remain paired to one of the original parental DNA strands. Replication of this molecule would yield a double-stranded symmetric molecule (Fig. 12G) that could lose or gain copies of SV40 DNA by homologous recombination to generate all of the various forms which are in equilibrium in the Ad5^{++}D1 population. An alternative model (2) involves the breakage of the parental molecule during replication. The broken end of DNA might then serve as a self-primer to allow the formation of a hairpin structure, which in turn could replicate to produce the symmetrically arranged hybrid genome.

By whatever mechanism they were originally generated, the structures of Ad5^{++}D1 and the adenovirus type 12-human DNA recombinant suggest the possibility that one could construct in vitro an adenovirus vector that might carry large segments of foreign DNA. By creating the appropriate restriction sites within the DNA sequences of the left end of adenovirus DNA, one might use such a vector to introduce up to 30 kilobases of a DNA of interest with high efficiency into mammalian cells.

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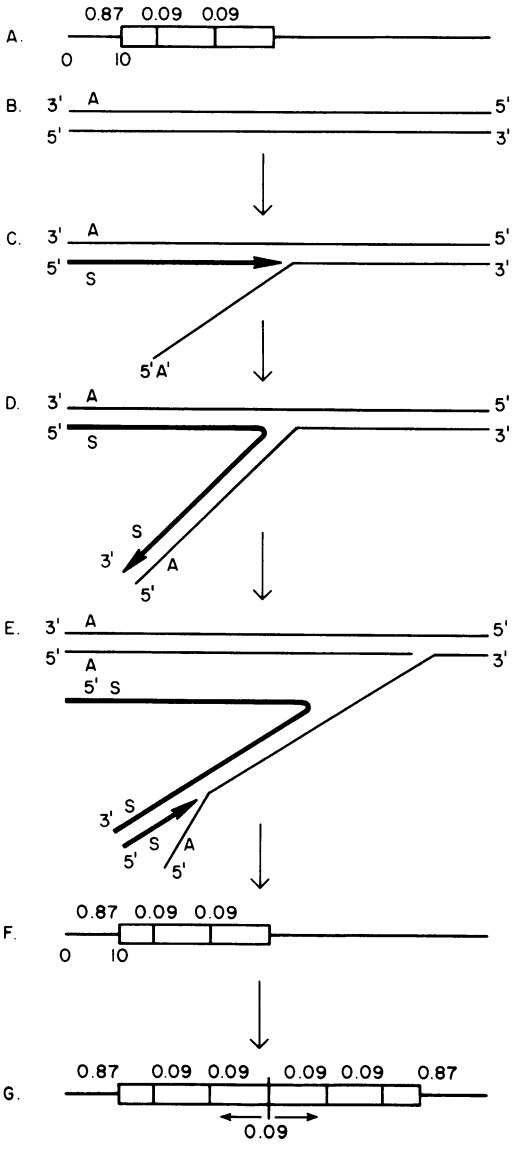

FIG. 12. Model proposing the origin of Ad5^{++}D1. (A) The structure represents a hypothetical adenovirus-SV40 hybrid showing the parental molecule from which Ad5^{++}D1 was generated, as illustrated. The molecule contains sequences of adenovirus type 5 DNA to nucleotide 3,534, joining the SV40 genome at nucleotide 1,102. The numbers above the structure represent SV40 map units, and those below are percentages of the adenovirus type 5 genome. (B) Parental molecule as a double-stranded DNA. (C) Replication of the molecule has begun. During replication the polymerase "jumped" strands and continued replicating by using the complementary strand as its template (D). Replication of this structure would generate a newly replicated parental molecule and a symmetric strand that would remain bound to the other parental strand (E). Further replication would generate a second parent molecule (F) and a new double-stranded symmetric recombinant molecule (G).

LITERATURE CITED

1. **Crumpacker, C. S., M. J. Levin, W. H. Wiese, A. M. Lewis, Jr., and W. P. Rowe.** 1970. Adenovirus type 2-simian virus 40 hybrid population: evidence for a hybrid deoxyribonucleic acid molecule and the absence of adenovirus-encapsidated circular simian virus 40 deoxyribonucleic acid. J. Virol. **6**:788–794.
2. **Daniell, E.** 1976. Genome structure of incomplete particles of adenovirus. J. Virol. **19**:685–708.
3. **Deuring, R., G. Klotz, and W. Doerfler.** 1981. An unusual symmetric recombinant between adenovirus type 12 DNA and human cell DNA. Proc. Natl. Acad. Sci. U.S.A. **78**:3142–3146.
4. **Grodzicker, T., and J. Sambrook.** 1979. Adenovirus-SV40 hybrids: a model system for expression of foreign sequences in an animal virus vector, p. 103–114. *In* J. K. Setlow, and A. Hollaender, (ed.), Genetic engineering, principles and methods. Plenum Press, New York.
5. **Hammarskjold, M. L., and G. Winberg.** 1980. Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. Cell **20**:787–795.
6. **Hammarskjold, M. L., G. Winberg, E. Norrby, and G. Wadell.** 1977. Isolation of incomplete adenovirus 16 particles containing viral and host cell DNA. Virology **82**:449–461.
7. **Hassell, J. A., E. Lukanidin, G. Fey, and J. Sambrook.** 1978. The structure and expression of two defective adenovirus 2-simian virus 40 hybrids. J. Mol. Biol. **120**:209–247.
8. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
9. **Kelly, T. J., Jr., and A. M. Lewis, Jr.** 1973. Use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J. Virol. **12**:643–652.
10. **Kelly, T. J., Jr., A. M. Lewis, Jr., A. S. Levine, and S. Siegel.** 1974. Structure of two adenovirus-simian virus 40 hybrids which contain the entire SV40 genome. J. Mol. Biol. **89**:113–126.
11. **Lonberg-Holm, K., and L. Philipson.** 1969. Early events of virus-cell interaction in an adenovirus system. J. Virol. **4**:323–328.
12. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
13. **Maxam, A., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
14. **Pettersson, U., and J. Sambrook.** 1973. Amount of viral DNA in the genome of cells transformed by adenovirus type 2. J. Mol. Biol. **73**:125–130.
15. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
16. **Stringer, J. R.** 1982. DNA sequence homology and chromosomal deletion at a site of SV40 DNA integration. Nature (London) **296**:363–366.
17. **Tooze, J.** 1982. Adenovirus-SV40 hybrids, p. 577–614. *In* Molecular biology of DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Van Roy, F., and W. Fiers.** 1978. Structure of the components of the adenovirus-simian virus 40 hybrid population of $Ad2^{++}$HEY and reciprocal molecular interactions. J. Mol. Biol. **126**:691–720.
19. **Westphal, H.** 1981. Genomic arrangement of an adenovirus-simian virus 40 hybrid virus, $Ad2^{+}ND_{4del}$. J. Virol. **40**:526–532.
20. **Williams, J.** 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. **9**:251–255.
21. **Winnacker, E. L.** 1978. Adenovirus DNA: structure and function of a novel replicon. Cell **14**:761–773.
22. **Zain, B. S., and R. J. Roberts.** 1978. Characterization and sequence analysis of a recombination site in the hybrid virus $Ad2^{+}ND1$. J. Mol. Biol. **120**:13–31.